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Applicant RICHARDSON, Peter et al	

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PATENT COOPERATION TREATY

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

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IMPORTANT NOTIFICATION

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PATENT COOPERATION TREATY

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(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

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(57) Abstract

The present invention relates to methods of detecting an RNA sequence by tagging the sequence with a unique random nucleotide sequence during reverse transcription. The unique nucleotide sequence is then utilized to selectively amplify the resulting DNA sequence. The present invention reduces the number of false positives obtained as a result of contaminating DNA.

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MODIFIED RNA TEMPLATE-SPECIFIC POLYMERASE CHAIN REACTION

The present application is a continuation-in-part application of Serial No. 07/504,591 filed April 5, 1990, which is hereby incorporated in its entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a method for detecting an RNA sequence. More specifically, the present invention relates to a method of amplifying an RNA sequence using a modification of the polymerase chain reaction.

2. Background Information

The polymerase chain reaction (PCR) method, developed by Perkin-Elmer-Cetus Corporation, is a popular and extraordinarily powerful technique for the amplification of DNA sequences. It has wide-ranging applications including molecular biology, medical diagnostics, genetics, forensics, and archeology [Saiki et al., Science 230, 1350 (1985); Saiki et al., Science 239, 487 (1985); Kogan et al., N. Engl. J. Med. 316, 656 (1987); Higuchi et al., Nature 332, 543 (1988); and Paabo et al., J. Biol. Chem. 264, 9709 (1989)]. When coupled with reverse transcription (RT-PCR), this technique can detect as few as 1 to 100 copies of a specific RNA from single cells or small numbers of cells. [Kawasaki et al., Proc. Natl. Acad. Sci. U.S.A. 85, 5698 (1988); Rappolee et al., Science 241, 708 (1988); Rappolee et al., Science 241, 1823 (1988); Sarkar et al., Science 244, 331 (1988); and Frohman et al., Proc. Natl. Acad. Sci. U.S.A. 85, 8998 (1988)].

Unfortunately, the exquisite sensitivity of this technique presents one of its severe shortcomings, false positives resulting from contamination with minute quantities of DNA [Kwok et al., Nature 339, 237 (1989); Sakar et al., Nature 343, 27 (1989)]. Potential sources of contaminating DNA may include: 1) endogenous sources such as small quantities of genomic DNA which may copurify with RNA, and 2) exogenous sources such as cDNA, plasmid DNA, or DNA fragments amplified in previous PCRs (i.e. carryover). While the frequency of false positives can be reduced somewhat by instituting and maintaining special techniques (e.g. premixing and aliquoting reagents; use of disposable gloves and positive displacement pipettes; and adding the experimental sample last), contamination still remains a major problem, especially when low abundance RNA transcripts are being sought [Kwok et al., Nature 339, 237 (1989); Lo, Y.-M., et al., Lancet 2, 699 (1988)].

Conventional RT-PCR amplifies equally well DNA derived from an RNA template or from a DNA template. Therefore, small quantities of contaminating DNA from virtually any source may easily result in false positives. Assuming approximately 4 pg of genomic DNA per mammalian haploid cell, and a sensitivity of 1 to 100 copies, conventional RT-PCR would result in false positives from only picogram quantities of contaminating genomic DNA.

It is possible to avoid false positives caused by amplification of genomic DNA which may copurify with RNA if the target sequence to be amplified by RT-PCR spans an intron. However, this experimental design is

not always possible since 1) some genes do not contain introns in convenient regions, and 2) the genomic structure of many target genes are not yet known.

In the laboratory of the present inventors, RT-PCR was recently used to detect small quantities of Xenopus insulin mRNAs in unfertilized eggs and early embryos. Despite the fact that numerous precautions were taken to exclude contamination of Xenopus insulin cDNAs which had been previously cloned in our laboratory [Shuldiner et al., J. Biol. Chem. 264,9428 (1989)], frequent false positives precluded meaningful interpretation of the experiments.

The present invention, RNA template-specific PCR (RS-PCR) and modified RS-PCR, provides methods of detecting minute quantities of RNA without the problems of false positives associated with RT-PCR. In the present methods the reduction in the frequency of false positives is achieved without sacrificing sensitivity obtained with conventional RT-PCR.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of detecting an RNA sequence which reduces the number of false positives resulting from DNA contamination in the sample (i.e., previously cloned cDNAs, genomic DNA or carryover of DNA amplified in previous PCRs). The present method increases the accuracy of the procedure without sacrificing sensitivity.

It is another object of the present invention to provide a method of detecting an RNA sequence which obviates the necessity to choose a target RNA sequence which spans an intron.

Various other objects and advantages of the present invention will be apparent from the following description of the invention and the drawings.

In one embodiment, the present invention relates to a method of detecting an RNA sequence. The method comprises:

i) reverse transcribing the RNA sequence from an oligonucleotide primer hybridized thereto which oligonucleotide primer (d_{17}-t_{30}) comprises:

a) on the 3' end thereof (segment d_{17}), a nucleotide sequence complementary to a region of the RNA sequence to be detected; and

b) on the 5' end thereof (segment t_{30}), a unique random nucleotide sequence or tag whereby a single stranded DNA sequence is produced which has at its 5' end the unique sequence;

ii) hybridizing, at a temperature high enough to preclude annealing of the d_{17} segment of the d_{17}-t_{30} primer to possible contaminating DNA, but low enough to allow annealing, an upstream oligonucleotide primer (u_{30}), to a region of said DNA sequence to which it is complementary, a predetermined distance upstream from t_{30};

iii) extending the primer (u_{30}) so that a DNA molecule is produced having at its 3' end a nucleotide sequence complementary to said unique nucleotide sequence (segment t_{30});

iv) denaturing the double-stranded DNA molecule produced in step (iii);

v) hybridizing, at a temperature high enough to preclude annealing of the d_{17} segment of the d_{17}-t_{30} primer to possible contaminating DNA, but low enough to

allow annealing the upstream PCR oligonucleotide primer (u_{30}) to the region of said DNA sequence to which it is complementary and,

hybridizing, at a temperature high enough to preclude annealing of the d_{17} segment of the d_{17}-t_{30} primer to possible contaminating DNA, but low enough to allow annealing of a PCR oligonucleotide primer (t_{30}) comprising all or a portion of said unique nucleotide sequence, to the 3' end of said DNA sequence to which it is complementary;

vi) extending the primers (u_{30}) and (t_{30}) thereby producing two DNA molecules; and

vii) detecting the presence or absence of the amplified DNA sequence;

wherein the d_{17} segment of the oligonucleotide primer d_{17}-t_{30} does not hybridize to contaminating DNA at the annealing temperature of the PCR, and oligonucleotide primer u_{30} and oligonucleotide primer t_{30} do hybridize to their appropriate DNA templates at the annealing temperature of the PCR.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows diagrammatically the RNA template-specific PCR method (RS-PCR).

FIGURE 2 compares the sensitivity of conventional reverse transcriptase - PCR (RT-PCR) and novel RS-PCR when beginning with an RNA template.

FIGURE 3 compares conventional RT-PCR and novel RS-PCR when DNA rather than RNA is used as a starting template to mimic DNA contamination.

FIGURE 4 shows the effect of changing the nucleotide sequence of the unique segment of oligonucleotide primer d_{20}-t_{21}.

FIGURE 5 shows schematically the modified RNA template-specific PCR.

FIGURE 6 compares conventional RT-PCR and modified RS-PCR. RT primer $d_{17t}30$: PCR primers t_{30} and u_{30} (lanes 1-4); RT primer $d_{30}t$: PCR primers d_{30} and u_{30} (lanes 6-9). Lane 0 is a HaeIII digest of PhiX174 DNA, while lane 5 is RS-PCR in the absence of any template.

FIGURE 7 shows PCR carryover contamination is ignored with modified RS-PCR. Lanes 1 and 3; RT primer d_{17}-t_{30}, PCR primers u_{30} and t_{30}. Lanes 2 and 4; RT primer $d_{16}t'_{30}$, PCR primers u_{30} and t'_{30}.

FIGURE 8 shows the region of Xenopus insulin RNA that was used as the target RNA to test the modified RS-PCR procedure. Reverse transcription primer d_{17}-t_{30} contained a 17 base sequence at its 3' end (segment d_{17}) that was complementary (antisense) to a region of Xenopus insulin RNA in the 3' untranslated region (nucleotides 404-420), and 30 bases at its 5' end (segment t_{30}) that were unique in sequence. Upstream primer u_{30} is identical (sense) to Xenopus insulin RNA in the coding region (nucleotides 59-88).

DETAILED DESCRIPTION OF THE INVENTION

The present invention, RNA template-specific PCR (RS-PCR) and modified RS-PCR, relates to a targeted amplification method which distinguishes RNA in the sample from contaminating DNA and amplifies only sequences derived from RNA. Minute quantities of cDNA, plasmid DNA or carryover DNA amplified in previous PCRs can be important sources of contamination when using conventional RT-PCR. The present invention reduces the number of false positives obtained as a result of contaminating DNA. Furthermore, the present invention

obviates the necessity of choosing a target RNA sequence which spans an intron in order to distinguish the reverse transcribed DNA from contaminating genomic DNA. In addition, the modified RS-PCR eliminates the need for removal of the primer after reverse transcription, such as by ultrafiltration.

The RS-PCR method of the present invention is shown schematically in Figure 1.

In the first step, a first oligonucleotide primer designated d_{20}-t_{21} in Figure 1 (advantageously, of about 41 nucleotides) is hybridized to the RNA sequence to be detected. Primer d_{20}-t_{21} comprises on the 3' end, a nucleotide sequence (advantageously, about 20 nucleotides) complementary to the 3' end of the RNA sequence whose presence is to be detected (segment d_{20}), and on the 5' end, a unique random nucleotide sequence or tag (advantageously, about 21 nucleotides) (segment t_{21}). While the 3' end of the primer hybridizes to the RNA sequence, the 5' end of the primer remains unhybridized as no complementary sequence exists within the sample.

Once primer d_{20}-t_{21} has been hybridized to the 3' end of the RNA sequence, reverse transcriptase is used to extend the primer. The resulting single -(-) stranded DNA segment is thus tagged at its 5' end with the unique sequence t_{21} of original primer d_{20}-t_{21}. This unique 5' sequence (t_{21}) distinguishes between DNA generated from the RNA-template and possible contaminating DNA.

It is preferable for the unique sequence to be composed of approximately equal amounts of each nucleotide (i.e. about 25% of each nucleotide).

Furthermore, it is preferable to choose a unique sequence which is unlikely to have significant secondary structure, and does not contain significant complementarity at its 3' end with the 3' end of the upstream primer (for example, primer u_{21}). The sequence can also be selected so as to contain a convenient restriction enzyme recognition site if desired. One skilled in the art can easily generate by computer appropriate sequences, 5'-GACAAGCTTCAGGTAATCGAT-3' and 5'-CCGAATTCTGTAGTCCGTCA-3' being two examples.

Prior to amplification, excess primer d_{20}-t_{21} is removed by ultrafiltration through a Centricon 100 device (Amicon, Danvers, MA) or similar device.

In the second and third steps of the present method, the DNA segment resulting from the previous step is amplified using the PCR technique (see U.S. Patents 4,683,202 and 4,683,195). Two oligonucleotide primers designated u_{21} and t_{21} in Figure 1 are utilized to amplify the DNA. Upstream oligonucleotide primer u_{21} (advantageously, about 21 nucleotides) comprises a nucleotide sequence complementary the single stranded DNA segment produced in Step 1, a predetermined distance upstream from primer $d_{20}t_{21}$. Oligonucleotide primer t_{21} (advantageously, about 21 nucleotides) comprises the unique nucleotide sequence with which the segment of DNA was tagged during reverse transcription. The two primers are added to the sample and the PCR is carried out.

In the second step of the present method (PCR cycle 1), primer u_{21}, which is complementary to a region of the single stranded DNA segment produced in Step 1, a predetermined distance upstream from primer

$d_{20}t_{21}$, hybridizes thereto and is extended therefrom creating a complementary strand of DNA which includes at its 3' end a sequence complementary to the unique sequence. Primer t_{21} is not utilized in the first PCR cycle since no complementary sequence is present in the sample. However, primer t_{21} is used in the second PCR cycle and all cycles thereafter.

In the third step of the present method, the double stranded DNA segment resulting from the first PCR cycle, is denatured prior to the second PCR cycle.

For the second cycle and all subsequent PCR cycles, primer u_{21} which is complementary to the 3' end of the single - (-) stranded DNA hybridizes thereto and is extended therefrom. At the same time, the primer t_{21} hybridizes to its complementary sequence at the 3' end of the single - (+) strand of DNA and is extended therefrom. All DNA synthesis occurs in the 5' to 3' direction.

The modified RS-PCR method of the present invention is shown schematically in Figure 5.

The modified RS-PCR method eliminates the need to remove the first oligonucleotide primer, designated d_{17}-t_{30} in Figure 5, by selecting oligonucleotide primers d_{17}-t_{30}, t_{30} and d_{30}, so that differential hydridization occurs under the PCR conditions. The primers are selected so that the d_{17}-t_{30} primer and the d_{30} and the t_{30} primers anneal under different temperatures.

In the first step, as with the RS-PCR method, a first oligonucleotide primer designated d_{17}-t_{30} in Figure 5 (advantageously, of about 47 nucleotides) is hybridized to the RNA sequence to be detected. Primer d_{17}-t_{30} comprises on the 3' end, a nucleotide sequence

(advantageously, about 17 nucleotides) complementary to the 3' end of the RNA sequence whose presence is to be detected (segment d_{17}), and on the 5' end, a unique random nucleotide sequence or tag (advantageously, about 30 nucleotides) (segment t_{30}).

The primer should be selected so that the length of the d segment is such that it will not anneal efficiently to any DNA contaminants at the elevated annealing temperatures used in Steps 2 and 3. One skilled in the art can easily generate by computer suitable d_{17}-t_{30} primers including, for example, 5'-gaacatcgatgacaagcttaggtatcgatatgatggaattgccttga-3' and 5'-cttatacggatatcctggcaattcggacttgcatgatggaattgcc-3'.

Once primer d_{17}-t_{30} has been hybridized to the RNA sequence, reverse transcriptase is used to extend the primer thereby creating a single -(-) stranded DNA segment which is tagged at its 5' end with the unique sequence, the t_{30} segment, of original primer. This unique 5' sequence, as with the RS-PCR method, distinguishes between DNA generated from the RNA-template and possible contaminating DNA.

In the second step of the present method (PCR cycle 1), oligonucleotide primer designated u_{30} in Figure 5 (advantageously, about 30 nucleotides) hybridizes to the single stranded DNA generated in Step 1 a predetermined distance upstream from primer d_{17}-t_{30}, and is extended therefrom creating a complementary strand of DNA which includes at its 3' end a sequence complementary to the unique sequence. The primer u_{30} comprises a nucleotide sequence complementary to the single stranded DNA segment produced in Step 1, a predetermined distance upstream from primer $d_{17}t_{30}$. The

annealing stage of the PCR cycle is carried out at a temperature high enough to preclude annealing of the d_{17} segment of the reverse transcription primer d_{17}-t_{30} a to contaminating DNA, but low enough to allow annealing of PCR primer u_{30}, for example temperatures of 42° C or greater.

In the third step of the present method, the double stranded DNA segment resulting from the first PCR cycle, is denatured prior to the second PCR cycle.

For the second cycle and all subsequent cycles, primer u_{30} which is complementary to the single - (-) stranded DNA hybridizes thereto and is extended therefrom. At the same time, a second oligonucleotide primer, designated t_{30} in Figure 5 (advantageously, about 30 nucleotides) is added to the sample. The primer t_{30} comprises the unique nucleotide sequence with which the segment of DNA was tagged during reverse transcription. When the primer is added to the sample it hybridizes to its complementary sequence at the 3' end of the single - (+) strand of DNA and is extended therefrom. The annealing stage of all PCR cycles is conducted at a temperature high enough to preclude annealing of the d_{17} segment of the reverse transcription primer d_{17}-t_{30} a to contaminating DNA, but low enough to allow annealing of PCR primers u_{30} and t_{30}.

With RS-PCR, sequences derived from RNA that are tagged with the unique sequence (t_{30}) during reverse transcription (step 1) are amplified preferentially during PCR (steps 2 and 3). The original RS-PCR method requires ultrafiltration after reverse transcription to remove excess RT primer [Shuldiner et al., Gene 91, 139

(1990)]. The modified RS-PCR cirumvents this step by increasing the length of the RT and PCR primers, and increasing the PCR annealing temperature. The primers are selected so that the RT primer, d_{17}-t_{30}, hybridizes to the RNA template under the reverse transcription conditions but does not hybridize to possible DNA contaminants under the PCR conditions.

The longer length of the u_{30} and t_{30} primers allows annealing to occur at an increased temperature, that is temperatures up to about 72° C. Annealing of the 17 base d_{17} segment of the RT primer d_{17}-t_{30} occurs efficiently during reverse transcription at 37°C, but not at the higher PCR annealing temperature. Thus, when Steps 2 and 3 are carried out at a temperature of 42° C or greater (preferably 65° or greater), remaining d_{17}-t_{30} primer does not anneal to possible DNA contaminants while the u_{30} and t_{30} primers will anneal and be extended.

In both RS-PCR methods described above, each cycle of PCR involves primer hybridization, extension to yield double stranded DNA and denaturation. After the first PCR cycle, both the (+) and (-) strands of the DNA serve as templates from which a new strand of DNA is created. This leads to logarithmic expansion of the tagged segment of DNA.

Contaminating DNA lacks the unique nucleotide sequence. Thus, during the PCR the 3' end of the single - (-) strand of DNA serves as a template for primer u_{21} or u_{30} (but the 3' end of the -(+) strand can not act as template for unique primer t_{21} or t_{30} since there is no complementary sequence). This allows only linear amplification which, as one skilled in the art

knows, does not produce enough DNA to result in a false positive when detecting the presence of the logarithmically amplified PCR product.

Potential contamination arising from carryover of PCR products from previous experiments in which a different unique sequence was used is virtually eliminated when the present invention is used. With the methods of the present invention, no false positives were observed in over 20 independent experiments. The criteria for selecting the unique sequence of the primer used for reverse transcription and subsequent PCR is that the sequence selected is not present in the sample i.e. is unique. Therefore, the sequence used can be changed periodically. Changing the unique sequence prevents amplification of carryover PCR products. Thus, the methods of the present invention are particularly useful in a clinical laboratory setting where many samples and automation make careful laboratory hygiene more difficult.

The present invention is as sensitive as the well known PCR and RT-PCR procedures. Therefore, the small quantity of RNA needed is not affected. However, the present invention has the advantage of being more accurate.

In the following non-limiting examples, a segment of Xenopus insulin RNA is amplified by the present methods. The methods are applicable to the amplification of other RNAs.

Examples

RS-PCR

Example 1: RNA Template-Specific Polymerase Chain Reaction (RS-PCR)

Xenopus insulin mRNA was amplified using novel RS-PCR, which involves first reverse transcribing Xenopus pancreatic RNA using an oligonucleotide 41-mer as a primer (oligonucleotide d_{20}-t_{21}) whose nucleotide sequence contained 20-bases at the 3'-end which were complementary to a region of Xenopus insulin mRNA (segment d_{20}), and 21-bases at the 5'-end which consisted of a unique random sequence selected by computer or similar method (segment t_{21}) (FIGURE 1) followed by PCR amplification of the DNA segment.

As a first step, total RNA from Xenopus pancreatic tissue was prepared by the guanidinium isothiocyanate method [Chirgwin et al., Biochemistry 18, 5294 (1979)]. RNA was reverse transcribed at 42°C for one hour in a 25 µl reaction mixture containing Tris-HCl (50 mM, pH adjusted to 8.7 at room temperature), NaCl (100 mM), $MgCl_2$ (6 mM), dithiothreitol (10 mM), dNTP's (1 mM each), RNasin (1 µl; Promega Biotec; Madison, WI), oligonucleotide d_{20}-t_{21} (5'-GACAAGCTTCAGGTATCGATTTGCATGATGGAATTGCCTTG-3'; 0.5 µM), and AMV-reverse transcriptase (10 units; Promega Biotec).

This reverse transcription step resulted in single-(-) stranded DNA which had been "tagged" at its 5' end with a unique 21-nucleotide sequence or tag (segment t_{21}).

After reverse transcription, the oligonucleotide primer d_{20}-t_{21} was efficiently removed (>99.9%) using a Centricon 100 ultrafiltration device (Amicon; Danvers, MA) according to manufacturer's recommendations. Then PCR was performed using as primers oligonucleotide t_{21}, a 21-mer containing the

same unique nucleotide sequence as in segment t_{21} of oligonucleotide d_{20}-t_{21} and oligonucleotide u_{21}, a 21-mer complementary to the first strand, 244 bp upstream from oligonucleotide t_{21}.

PCR amplification was performed in a 50 µl reaction volume containing Tris-HCl (10 mM, pH adjusted to 8.3 at room temperature), KCl (50 mM), $MgCl_2$ (1.5 mM), gelatin (0.01%), dNTP's (200 µM each), oligonucleotide t_{21} (5'-GACAAGCTTCAGGTAATCGAT-3'; 0.5, µM), oligonucleotide u_{21} (5'-GAGGCTTCTTCTACTACCCTA-3'; 0.5 µm) and Taq polymerase (1 units; Perkin Elmer-Cetus Corp., Emeryville, CA). The reaction mixture was covered with paraffin oil (approximately 50 µl), heated to 94°C for 5 minutes, followed by PCR (45-60 cycles). Each cycle consisted of annealing (55°C, 1.5 min), extension (72°C, 1.5 min) and denaturation (94°C, 1 min) except for the last cycle, in which the extension time was increased to 15 minutes to insure completeness of extension.

Twenty microliters of the reaction mixtures were loaded onto a composite gel consisting of 1% agarose and 2% Nusieve GTG (FMC Bioproducts; Rockland, ME) in Tris-borate-EDTA buffer, electrophoresed, stained with ethidium bromide, and visualized by UV transillumination.

Since logarithmic amplification is dependent upon nucleotide sequences corresponding to d_{20}, t_{21} and u_{21}, only sequences derived from Xenopus insulin RNA which had been reverse transcribed with oligonucleotide d_{20}-t_{21} were amplified logarithmically, and contaminating DNA, which lacks the oligonucleotide t_{21} sequence was not amplified logarithmically.

Example 2: Comparison of novel RS-PCR and conventional RT-PCR using an RNA template.

To test whether novel RS-PCR was as sensitive as conventional RT-PCR, Xenopus pancreatic RNA which had been reverse transcribed with oligonucleotide d_{20}-t_{21} and ultrafiltered, was subjected to either conventional RT-PCR (oligonucleotides d_{20} and u_{21}), or novel RS-PCR (oligonucleotides t_{21} and u_{21}). PCR with either of these two oligonucleotide pairs resulted in similar sensitivity (FIGURE 2).

Xenopus pancreatic RNA (1 ng) was reverse transcribed and ultrafiltered according to the methods of Example 1.

For the conventional RT-PCR, 60 cycles of the PCR were performed on serial ten-fold dilutions of reverse transcribed and ultrafiltered pancreatic RNA with oligonucleotide primers d_{20} and u_{21} (FIGURE 2, lanes 1-5) using the conditions described in Example 1.

For the novel RS-PCR comparison, identical serial dilutions of the reverse transcribed and ultrafiltered pancreatic RNA was amplified by PCR using oligonucleotide primers t_{21} and u_{21} (FIGURE 2, lanes 6-9).

The predicted 244-bp and 265-bp amplified bands observed on the ethidium bromide-stained gel hybridized strongly to a radiolabeled full-length Xenopus insulin cDNA probe [Southern J. Mol. Biol. 98, 503 (1975)].

PCR with either the oligonucleotide pair d_{20} and u_{21} or the pair t_{21} and u_{21} resulted in similar sensitivity. Conventional RT-PCR with or without

removal of excess oligonucleotide d_{20}-t_{21} by Centricon 100 ultrafiltration resulted in similar sensitivity, as did reverse transcription with oligonucleotide d_{20} as the primer rather than oligonucleotide d_{20}-t_{21}. These results suggest that neither Centricon 100 ultrafiltration or reverse transcription using an oligonucleotide with a random 21-nucleotide overhang at its 5' end result in a significant decrease in sensitivity.

Example 3: Comparison of novel RS-PCR and conventional RT-PCR using a DNA template

By contrast to the Example 2 where the sensitivity of the reaction was not affected by the use of the unique nucleotide sequence, novel RS-PCR was approximately 10 to 1000-fold less affected by the presence of DNA contaminants (i.e., Xenopus insulin cDNA) than conventional RT-PCR even after 60 cycles (FIGURE 3).

Full-length Xenopus insulin cDNA (300 pg) was "reverse transcribed" with oligonucleotide d_{20}-t_{21}, excess oligonucleotide d_{20}-t_{21} removed by ultrafiltration, and PCR (60 cycles) was accomplished as described in the above Examples. Results of the conventional RT-PCR performed on serial ten-fold dilutions of the "reverse transcribed" and ultrafiltered Xenopus insulin cDNA using oligonucleotides d_{20} and u_{21} is shown in FIGURE 3, lanes 1-5. Novel RS-PCR of identical serial ten-fold dilutions of "reverse transcribed" and ultrafiltered Xenopus insulin cDNA using oligonucleotides t_{21} and u_{21} is shown in lanes 6-10 of the same figure.

In theory, with the RS-PCR method, only RNA that had been primed with oligonucleotide d_{20}-t_{21} during RT should have been amplified during PCR. However, it was found that when relatively large quantities of DNA template (>10 pg or approximately 1 x 10^7 molecules) were used, detectable amplification was observed (lane 6 in Fig. 3). It has been determined from separate experiments that this phenomenon was caused by two mechanisms; i) at relatively high DNA concentrations, RT acted as a DNA polymerase and incorporated oligonucleotide d_{20}-t_{21} into the so-called first strand, and ii) the minute quantities of oligonucleotide d_{20}-t_{21} that remained behind after ultrafiltration incorporated into DNA during early PCR cycles which could then be amplified efficiently in RS-PCR.

Example 4: Effect of changing the sequence of the unique segment t_{21} of oligonucleotide d_{20}-t_{21} on conventional RT-PCR and novel RS-PCR.

In order to evaluate the ability of the RS-PCR method to eliminate problems of carryover contamination of amplified DNA from previous RS-PCR experiments which had been tagged with a different unique sequence t_{21} the following experiment was conducted.

Xenopus pancreatic RNA (1 ng) was reversed transcribed with either oligonucleotide 41-mer d_{20}-t_{21} (FIGURE 4, lanes 1, 2, 3 and 7), or oligonucleotide 41-mer d_{20}-t'_{21} (5'-CCGAATTCTGTAGTCCGTCATTGCAGATGGAATTGCCTTG-3') (FIGURE 4, lanes 4-6). After ultrafiltration, PCR (45 cycles) was accomplished as described in the previous Examples using oligonucleotide pairs t_{21} and u_{21} (FIGURE 4, lanes

1 and 4), t'_{21} (5'-CCGAATTCTGTAGTCCGTCA-3') and u_{21} (FIGURE 4, lanes 2 and 5), d_{20} and u_{21} (FIGURE 4, lanes 3 and 6), or t_{21} and u'_{21} (5'-TGACCTTTCCAGCACTTATC-3') (FIGURE 4, lane 7).

As expected, the RNA that had been reversed transcribed with oligonucleotide d_{20}-t_{21} was amplified only when oligonucleotide t_{21} was used during PCR, but not when an unrelated unique 21-mer (oligonucleotide t'_{21}) was used. Conversely, reverse transcription of Xenopus pancreatic RNA with oligonucleotide d_{20}-t'_{21}, could only be amplified by the corresponding unique 21-mer, oligonucleotide t'_{21}, and not by the unrelated random 21-mer, oligonucleotide t_{21}. As expected, when conventional RT-PCR was used (i.e. oligonucleotide d_{20}), amplification occurred regardless of whether reverse transcription primers d_{20}-t_{21} or d_{20}-t'_{21} were used.

MODIFIED RS-PCR

Example 5: Modified RS-PCR

Oligonucleotides were synthesized on a Coder 300 automated DNA synthesizer (E.I. Du Pont Company; Wilmington, DE), and purified with NENsorb Prep columns (New England Nuclear; Boston, MA) according to the manufacturer's directions (see Table 1 below). Xenopus insulin (sense) RNA was prepared by ligating an 890 bp Xenopus insulin cDNA [Shuldiner et al., J. Biol. Chem. 264, 9428 (1989)] into pSP71 (Promega Biotec; Madison, WI).

After linearization of the recombinant plasmid with BglII, T7 RNA polymerase (Promega Biotec) was used for in vitro transcription to generate Xenopus insulin (sense) RNA. The RNA was purified by oligo-dT cellulose chromatography (Bethesda Research

Laboratories). Only full-length RNA was retained by the column since the 3' end contained a long poly-A tail. The RNA was quantitated by UV absorbance at 260 nm.

RNA was diluted to the appropriate concentration in water containing yeast tRNA (100 µg/ml) (Bethesda Research Laboratories). DNA templates used to demonstrate RNA specificity were either a double-stranded 890 bp Xenopus insulin cDNA insert [Shuldiner et al., J. Biol. Chem. 264, 9428 (1989)], or a 377 bp Xenopus insulin RS-PCR product that had been subjected to ultrafiltration with a Millipore-MC-100 device (Millipore; Bedford, MA) to remove excess primers. DNA templates were quantitated by comparison to a known quantity of a HaeIII digest of PhiX174 (Bethesda Research Laboratories) after agarose gel electrophoresis.

Table I. Primer sequences used to compare improved RS-PCR to conventional RT-PCR.

Primer	Sequence
$d_{17}t_{30}$	5'-GAACATCGATGACAAGCTTAGGTATCGATATGATGGAATTGCCTTGA-3'
t_{30}	5'-GAACATCGATGACAAGCTTAGGTATCGATA-3'
$d_{16}t'_{30}$	5'-CTTATACGGATATCCTGGCAATTCGGACTTGCATGATGGAATTGCC-3'
t_{30}	5'-CTTATACGGATATCCTGGCAATTCGGACTT-3'
d_{30}	5'-GCATGATGGAATTGCCTTGAAGGTGCCTTG-3'
u_{30}	5'-ATGCAGTGTCTGCCCCTGGTTCTTGTCCTC-3'

Reverse transcription of serial ten-fold dilutions of Xenopus insulin RNA (10^7 to 10^4 copies) was accomplished at 37° C in a final volume of 20 µl containing KCl (50 mM), Tris-HCl (10 mM; pH 8.3 at 25° C), $MgCl_2$ (1.5 mM), gelatin (0.01 mg/ml), dNTPs (200 µM each), RNasin (40 U; Promega Biotec), AMV-reverse transcriptase (7 U; Promega Biotec), and primer d_{17}-t_{30} (0.5 µM).

Primer d_{17}-t_{30} (Table I) was a 47-mer whose sequence contained 17 bases at its 3'-end that were complementary to a region of Xenopus insulin mRNA, designated segment d_{17}, and 30 bases at its 5'-end that were unique in sequence, designated segment t_{30}. Thus, reverse transcription yields single-stranded DNA that contains a unique 30 base "tag" (segment t_{30}) at its 5' end (FIGURE 5).

The second strand was synthesized during the first cycle of PCR in which 5 µl of the RT reaction mixture from step 1 was used directly in a final volume of 50 µl containing KCl (50 mM), Tris-HCl (10 mM; pH 8.3 at 25° C), $MgCl_2$ (1.5 mM), gelatin (0.01 mg/ml), dNTPs (200 µM each), upstream primer u_{30} (0.5 µM), downstream primer t_{30} (0.5 µM) and Taq polymerase (1.5 U; Perkin Elmer-Cetus; Emeryville, CA).

Upstream (sense) primer u_{30} was a 30-mer corresponding to Xenopus insulin cDNA that was 347 bp upstream from the sequence corresponding to segment d_{17}, while downstream primer t_{30} was a 30-mer whose sequence was identical to segment t_{30} of RT primer d_{17}-t_{30} (see Table I). With these primers, sequences derived from RNA that had been tagged with unique

sequence (t_{30}) during r verse transcription were amplified logarithmically preferentially, while contaminating DNAs, lacking the unique tag, were not amplified logarithmically (FIGURE 5) [Shuldiner et al., Gene 91, 139 (1990)].

After covering the PCR reaction mixture with parafin oil (approximately 50 µl), 35 cycles of PCR were performed, each cycle consisting of denaturation (94° C, 1 min) and annealing/extension (70° C, 2 min). In the first cycle, the denaturation time was increased to 5 min, and in the last cycle, the annealing/extension time was increased to 10 min to ensure completeness of the extension.

Twenty microliters of the PCR reaction mixture was electrophoresed on a composite gel consisting of 1% agarose (Bethesda Research Laboratories) and 2% Nuseive GTG (FMC Bioproducts; Rockland, ME). DNA was visualized by ethidium bromide staining and UV transillumination.

Example 6: Comparison of modified RS-PCR and conventional RT-PCR

To compare the sensitivity of modified RS-PCR to conventional RT-PCR, serial ten-fold dilutions of Xenopus insulin RNA (10^7 to 10^4 molecules) were amplified using either modified RS-PCR (RT primer d_{17}-t_{30}; PCR primers u_{30} and t_{30}) (FIGURE 6, panel a, lanes 1-5), or conventional RT-PCR (RT primer d_{30}; PCR primers u_{30} and d_{30}) (FIGURE 6, panel a, lanes 6-9).

Modified RS-PCR was equally sensitive to conventional RT-PCR when beginning with an RNA template. By contrast, when Xenopus insulin double-

stranded DNA (10^7 copies) was used as starting template to mimic DNA contamination, conventional RT-PCR resulted, as expected, in a strong signal (FIGURE 6, panel b, lane 11), while the modified RS-PCR method virtually ignored theDNA template (FIGURE 6, panel b, lane 10). When larger amounts of DNA were used (i.e., > 10^8 copies), a faint signal was detected with RS-PCR [Shuldiner et al., Gene 91, 139 (1990)].

To mimic RS-PCR carryover contamination, RS-PCR was performed with two 377 bp Xenopus insulin RS-PCR products (approximately 10^8 copies) that were identical to each other except each contained a different unique tag (sequence t_{30} or t'_{30} (Table I and FIGURE 7).

Double-stranded Xenopus insulin DNA containing either tag sequences t_{30} (FIGURE 7, lanes 1 and 2), or t'_{30} (FIGURE 7, lanes 3 and 4) were subjected to improved RS-PCR as described expect 30 cycles were performed. Amplification of each DNA template occurred efficiently when the primers matched the unique tag present in the PCR product (FIGURE 7 lanes 1 and 4). However, when RS-PCR primers were used that did not match the unique tag present in the PCR product, no amplification occurred (FIGURE 7, lanes 2 and 3). Thus, carryover contamination of RS-PCR products in which one unique tag was used is virtually eliminated when RS-PCR is performed with a different unique tag.

* * * * *

The entire contents of all publications cited hereinabove are hereby incorporated by

referenc .

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of detecting an RNA sequence comprising the steps of:

i) reverse transcribing the RNA sequence from an oligonucleotide primer hybridized thereto which oligonucleotide primer (d_{20}-t_{21}) comprises:

a) on the 3' end thereof, a nucleotide sequence complementary to the 3' end of the RNA sequence (segment d_{20}); and

b) on the 5' end thereof, a unique random nucleotide sequence or tag whereby a single stranded DNA sequence is produced which has at its 5' end said unique sequence (segment t_{21});

ii) removing excess oligonucleotide primer (d_{20}-t_{21});

iii) hybridizing an upstream oligonucleotide primer (u_{21}) complementary to the 3' end of said DNA sequence thereto;

iv) extending the primer (u_{21}) so that a DNA molecule is produced having at its 3' end a nucleotide sequence complementary to said unique nucleotide sequence;

v) denaturing the double-stranded DNA molecule produced in step (iv);

vi) hybridizing the oligonucleotide primer (u_{21}) to the 3' end of said DNA sequence to which it is complementary and hybridizing an oligonucleotide primer (t_{21}) comprising all or a portion of said unique nucleotide sequence, to the 3' end of said DNA sequence to which it is complementary;

vii) extending the primers (u_{21}) and (t_{21}) thereby producing two DNA molecules; and

viii) detecting the presence or absence of the amplified DNA sequence.

2. The method according to claim 1 further comprising before step (viii) repeating steps v-vii multiple times.

3. The method according to claim 1 wherein said oligonucleotide primer (d_{20}-t_{21}) is about 41 nucleotides in length.

4. The method according to claim 1 wherein said oligonucleotide primers (u_{21}) and (t_{21}) are each about 21 nucleotides in length.

5. The method according to claim 1 wherein said unique random nucleotide sequence is comprised of approximately 25% of each nucleotide.

6. The method according to claim 1 wherein said RNA sequence is a segment of mRNA.

7. The method according to claim 6 wherein said segment of mRNA is a segment of insulin mRNA.

8. The method according to claim 7 wherein said insulin mRNA is Xenopus insulin mRNA.

9. The method according to claim 1 wherein said unique nucleotide sequence is 5'-GACAAGCTTCAGGTAATCGAT-3'.

10. The method according to claim 1 wherein said unique nucleotide sequence is 5'-CCGAATTCTGTAGTCCGTCA-3'.

11. The method according to claim 1 wherein said segment of RNA is contaminated with DNA.

12. A method of detecting an RNA sequence comprising the steps of:

i) reverse transcribing the RNA sequence from an oligonucleotide primer hybridized thereto which oligonucleotide primer (d_{17}-t_{30}) comprises:

a) on the 3' end thereof (segment d_{17}), a nucleotide sequence complementary to a region of the RNA sequence; and

b) on the 5' end thereof (segment t_{30}), a unique nucleotide sequence or tag whereby a single stranded DNA sequence is produced which has at its 5' end said unique sequence;

ii) hybridizing an upstream oligonucleotide primer (u_{30}), complementary to said DNA sequence, to a region of said sequence, at a temperature selected so that said d_{17} segment does not anneal to contaminating DNA but so that said primer u_{30} does anneal;

iii) extending the primer (u_{30}) so that a DNA molecule is produced having at its 3' end a nucleotide sequence complementary to said unique nucleotide sequence (segment t_{30});

iv) denaturing the double-stranded DNA molecule produced in step (iii);

v) hybridizing the oligonucleotide primer (u_{30}) to said DNA sequence to which it is complementary and hybridizing an oligonucleotide primer (t_{30}) comprising all or a portion of said unique nucleotide sequence, to a region of said DNA sequence to which it is complementary,

wherein said hybridization is carried out at a temperature selected so that said d_{17} segment does not anneal to contaminating DNA but so that said primers u_{30} and t_{30} do anneal;

vi) extending the primers (u_{30}) and (t_{30}) thereby producing two DNA molecules; and

vii) detecting the presence or absence of the amplified DNA sequence;

13. The method according to claim 12 wherein said hybridization is carried out at a temperature 42° C or greater.

14. The method according to claim 12 further comprising before step (vii) repeating steps iv-vi multiple times.

15. The method according to claim 12 wherein said hybridization in steps ii and v occurs at a temperature between 65° and 72° C.

16. The method according to claim 12 wherein said oligonucleotide primer (d_{17}-t_{30}) is about 47 nucleotides in length.

17. The method according to claim 12 wherein said oligonucleotide primers (u_{30}) and (t_{30}) are each about 30 nucleotides in length.

18. The method according to claim 12 wherein said unique random nucleotide sequence is comprised of approximately 25% of each nucleotide.

19. The method according to claim 12 wherein said RNA sequence is a segment of mRNA.

20. The method according to claim 18 wherein said segment of mRNA is a segment of insulin mRNA.

21. The method according to claim 19 wherein said insulin mRNA is Xenopus insulin mRNA.

RNA Template-Specific PCR (RS-PCR)

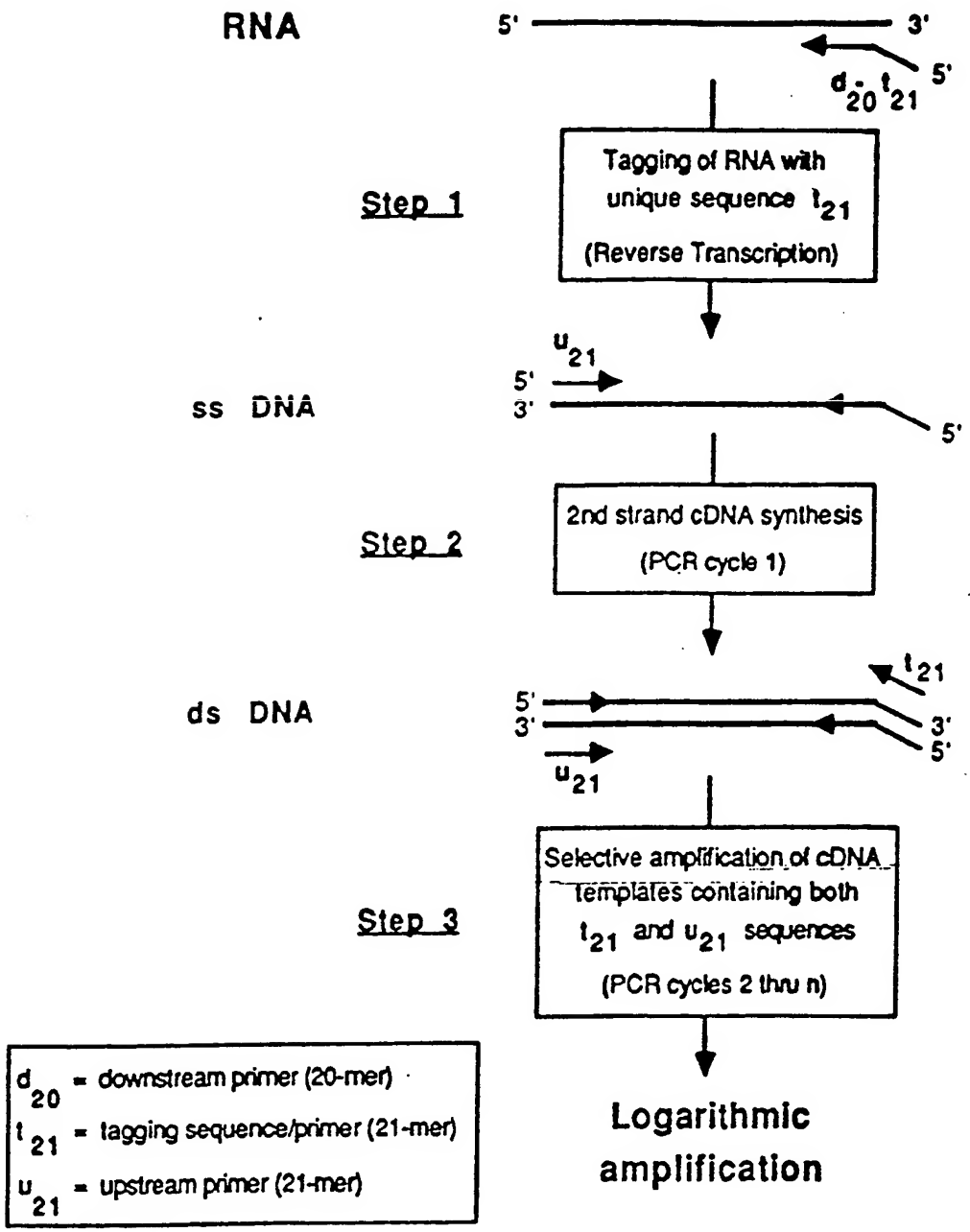


FIGURE 1

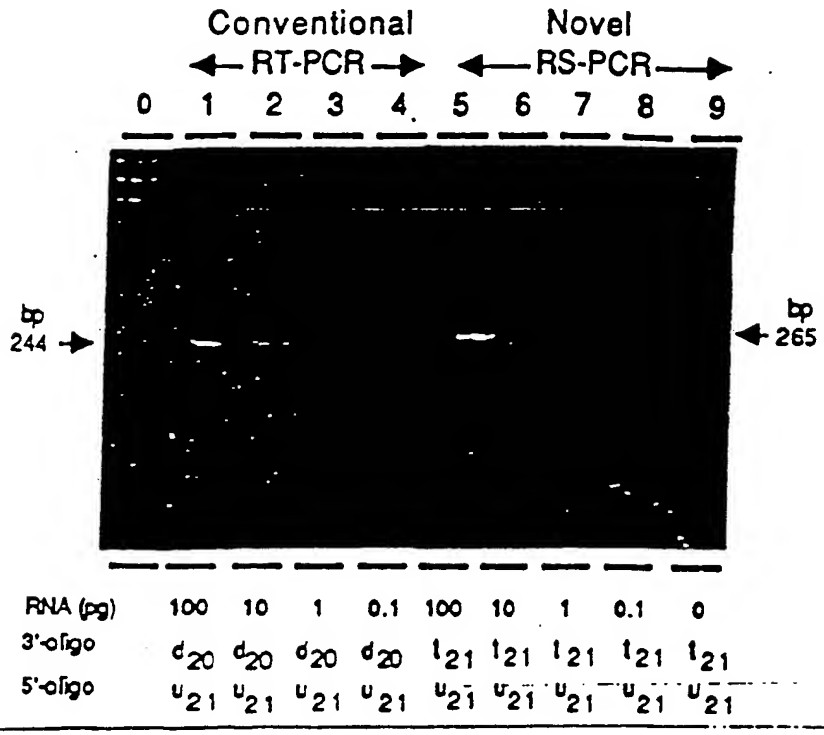

Conventional
RT-PCR
Novel
RS-PCR
0 1 2 3 4 5 6 7 8 9
bp 244
bp 265
RNA (pg) 100 10 1 0.1 100 10 1 0.1 0
3'-oligo d_{20} d_{20} d_{20} d_{20} t_{21} t_{21} t_{21} t_{21} t_{21}
5'-oligo u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21}

FIGURE 2

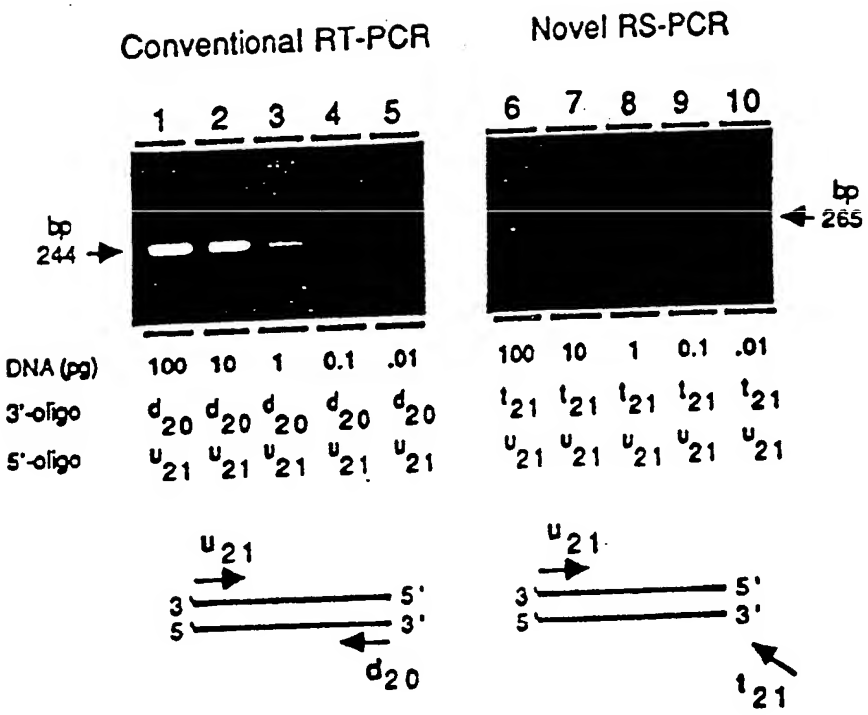
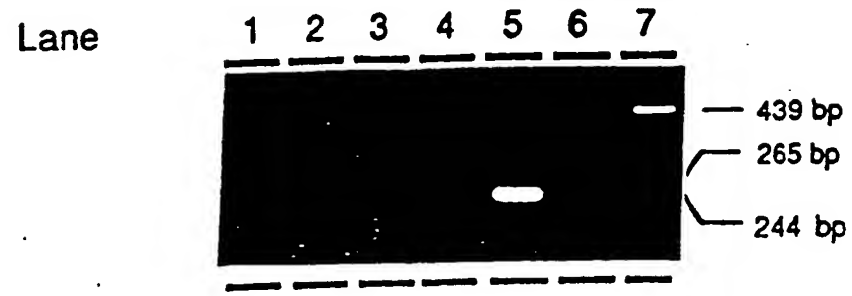

Conventional RT-PCR
Novel RS-PCR
1 2 3 4 5
6 7 8 9 10
bp
244
bp
265
DNA (pg) 100 10 1 0.1 .01 100 10 1 0.1 .01
3'-oligo d_{20} d_{20} d_{20} d_{20} d_{20} t_{21} t_{21} t_{21} t_{21} t_{21}
5'-oligo u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21} u_{21}
u_{21}
3
5
5'
3'
d_{20}
u_{21}
3
5
5'
3'
t_{21}

FIGURE 3

RS-PCR: Effect of changing the unique random sequence



RT primer tag	t_{21}	t_{21}	t_{21}	t'_{21}	t'_{21}	t'_{21}	t_{21}
3'-PCR oligo	t_{21}	t'_{21}	d_{20}	t_{21}	t'_{21}	t'_{21}	t_{21}
5'-PCR oligo	u_{21}	u_{21}	u_{21}	u_{21}	u_{21}	u_{21}	u'_{21}
RNase (mg/ml)	0	0	0	0	0	1	0

FIGURE 4

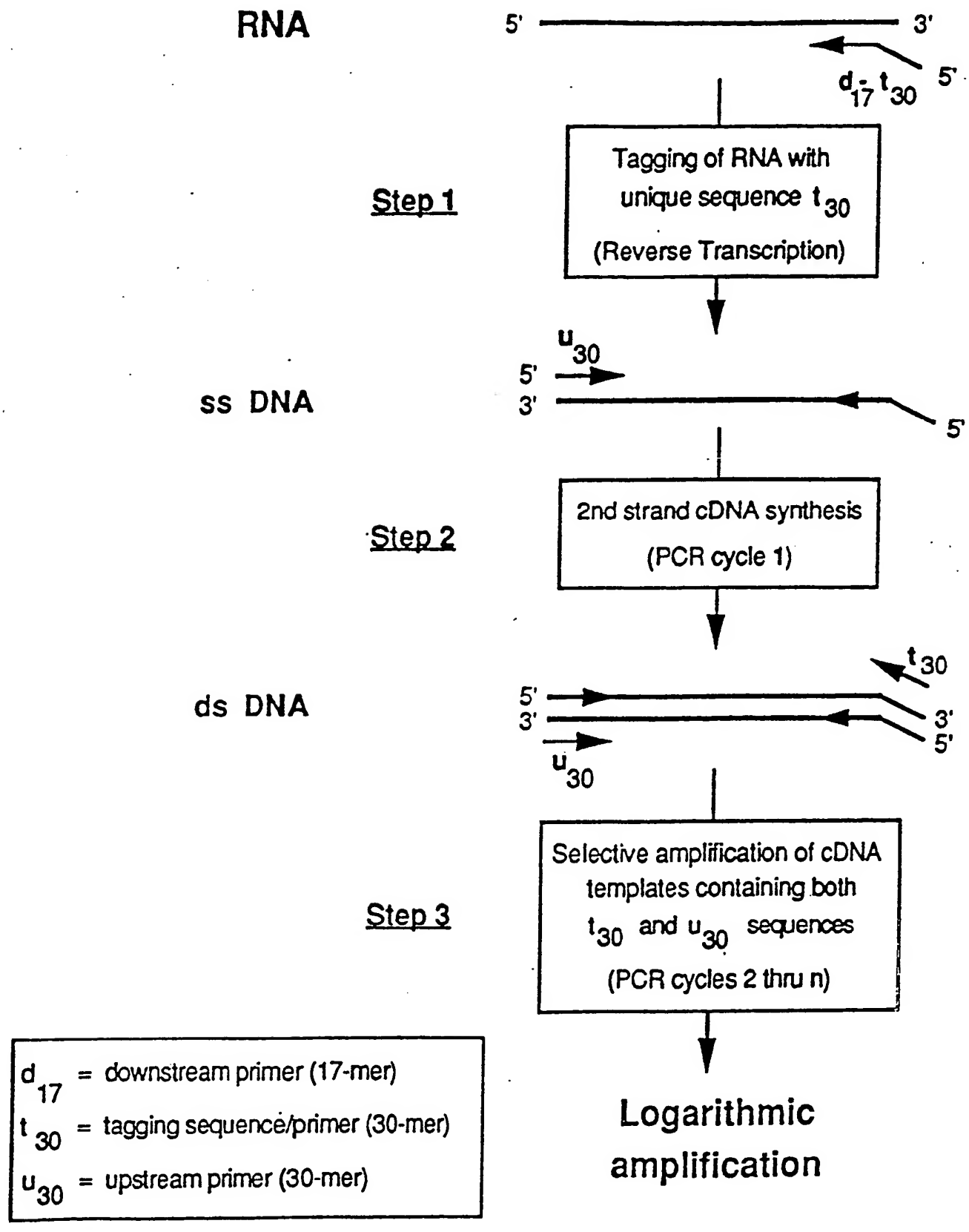


FIGURE 5

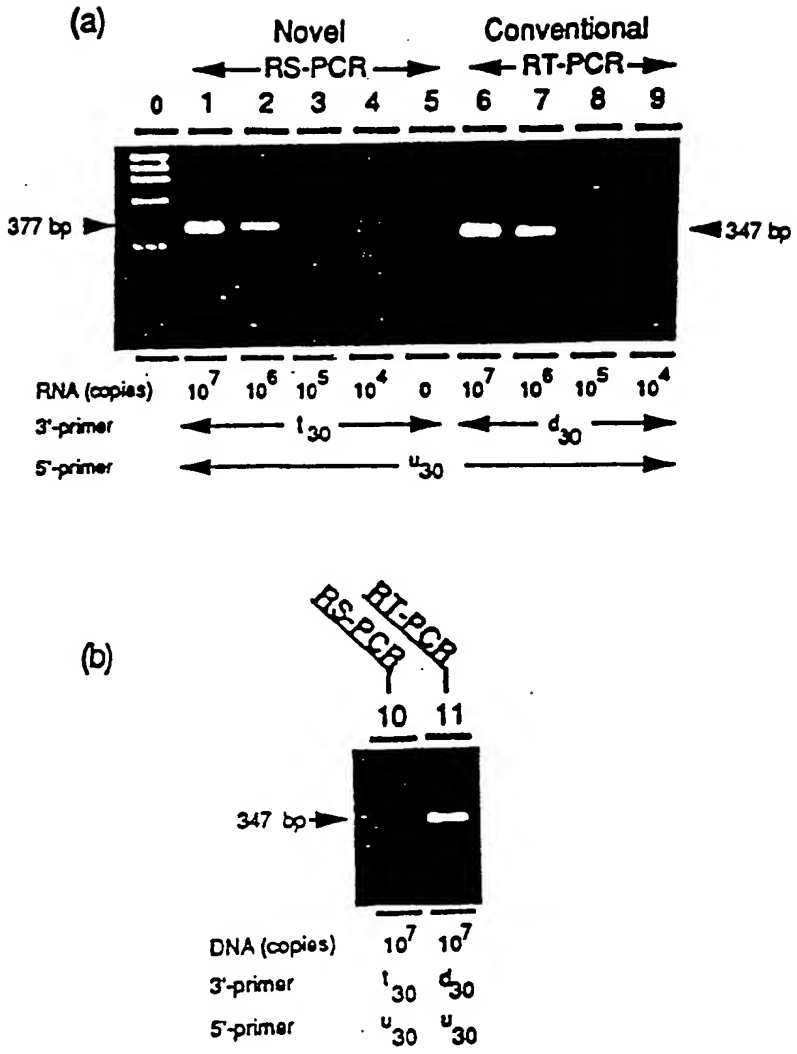


FIGURE 6

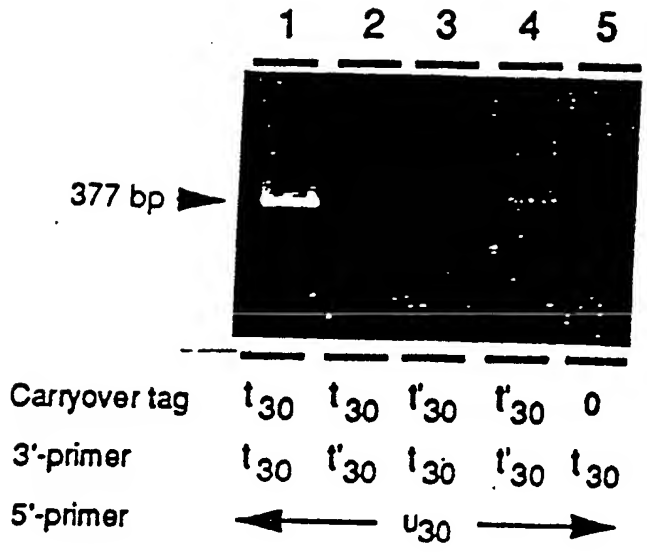

1 2 3 4 5
377 bp
Carryover tag t_{30} t_{30} t'_{30} t'_{30} 0
3'-primer t_{30} t'_{30} t_{30} t'_{30} t_{30}
5'-primer u_{30}

FIGURE 7

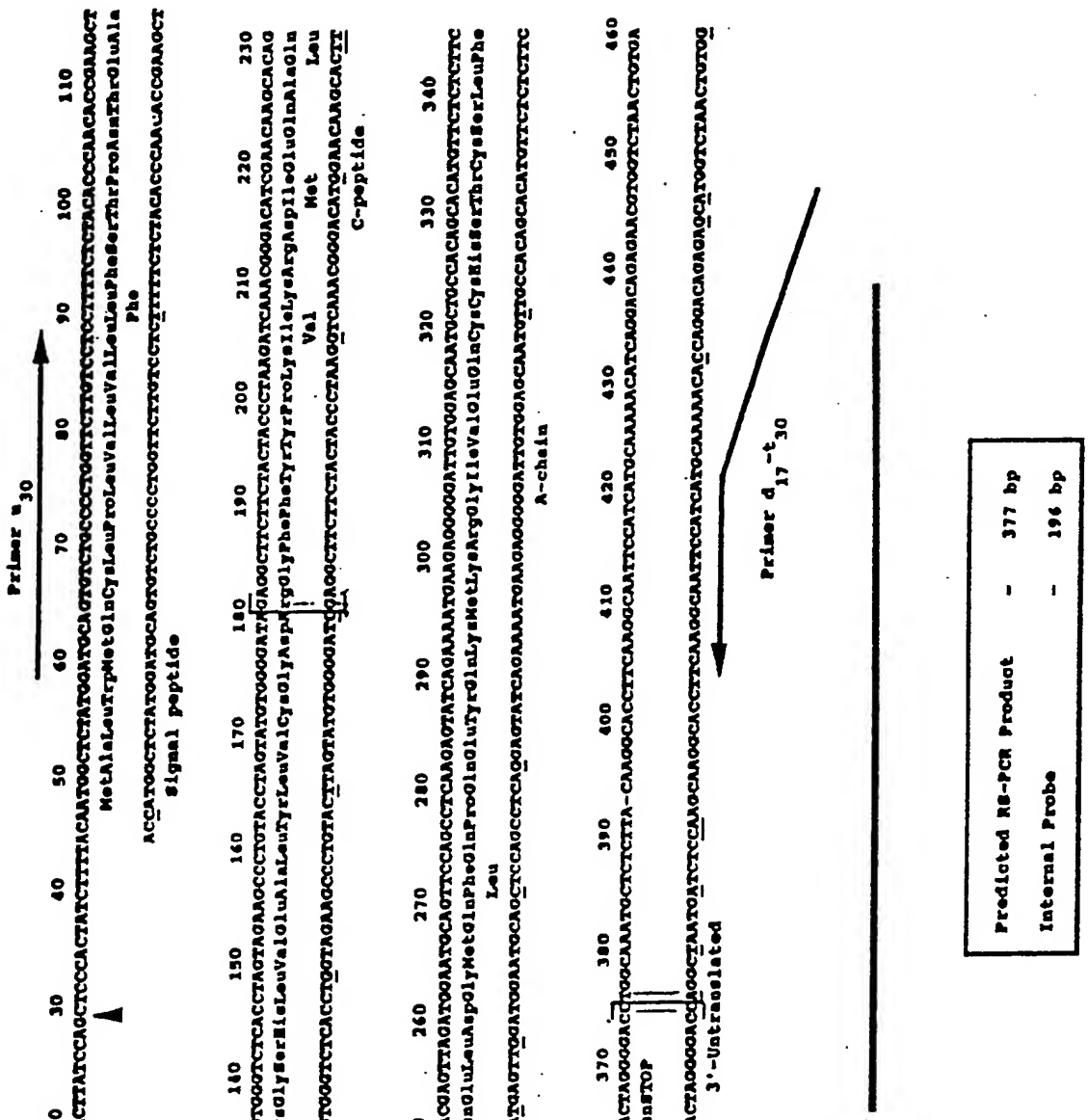


FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02211

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68; C12P 19/34; G01N 33/48, 33/566; C07H 15/12
US CL: 435/6, 91; 436/94, 501; 536/26, 27, 28, 29; 935/77, 78

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
USCL	435/6, 91; 436/94, 501; 536/26, 27, 28, 29; 935/77, 78

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,683,195 (MULLIS et al) 28 July 1987, See column 6, lines 62-64, column 7, lines 66-68; column 10, lines 10-13; column 29, example 9.	1-21
Y	Science, Vol. 243, issued 13 January 1989, E. Y. LOH et al., "Polymerase Chain Reaction with Single-Sided Specificity: Analysis of T Cell Receptor Delta Chain", pages 217-220; see page 218, Figure 1.	1-21

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 June 1991	05 AUG 1991

International Searching Authority	Signature of Authorized Officer
ISA/US	Stephanie W. Zitomer, Ph.D.

Form PCT/ISA/210 (second sheet) (rev. 11-87)

14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
911L PCT 477		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/06887	19/07/2000	19/07/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant CAMBRIDGE UNIVERSITY TECHNICAL SERVICES et al. LTD		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 10 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
13/02/2001	12.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer BROCHADO GARGANTA, M Telephone No. +49 89 2399 8935

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/06887

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-67	as originally filed				

Claims, No.:

1-138	as originally filed				
139-142	as received on	08/10/2001	with letter of	05/10/2001	

Drawings, sheets:

1/9-9/9	as originally filed				

Sequence listing part of the description, pages:

1-15, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☒ contained in the international application in written form.
   - ☒ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence

listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	36,38,41-44,111-135
	No:	Claims	1-35,37,39,40,45-110,136-141
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-141
Industrial applicability (IA)	Yes:	Claims	1-141
	No:	Claims	

2. Citations and explanations
   see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I
Basis of the report

5. The amendments do not meet the requirements of Article 34(2)(b) PCT, as they introduce additional subject-matter, which extends beyond the content of the application as filed.

New filed claim 142 relates to a heeled primer population, wherein each primer comprises a heel region of at least 15 nucleotides in length and a RNA polymerase promoter site. In the application as filed there is only a reference to a heel region of 15 to 22 nucleotides in length, which is not the same that at least 15 nucleotides. Moreover, it is not clear from what the variable sequence is differing (see page 10, lines 18-20). Thus, claim 142 will not be considered for the examination on novelty, inventive step and industrial applicability.

Re Item V
Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

(A) ZHAO ET AL.: '3'-END cDNA POOL SUITABLE FOR DIFFERENTIAL DISPLAY FROM A SMALL NUMBER OF CELLS' BIOTECHNIQUES, vol. 24, May 1998 (1998-05), pages 842-852

(B) TELENIUS H ET AL: 'DEGENERATE OLIGONUCLEOTIDE-PRIMED PCR: GENERAL AMPLIFICATION OF TARGET DNA BY A SINGLE DEGENERATE PRIMER' GENOMICS,US,ACADEMIC PRESS, SAN DIEGO, vol. 13, 1992, page 718-725

(C) ZHAO ET AL.: 'NEW PRIMER STRATEGY IMPROVES PRECISION OF

DIFFERENTIAL DISPLAY' BIOTECHNIQUES, vol. 18, no. 5, 1995, pages 842- 850

2. Novelty

2.1 The subject-matter of claim 1, relating to a method to increase the number of nucleotide sequences corresponding to the mRNA species present in a sample, is not new in the sense of Article 33(2) PCT, because such a method is already disclosed in document A.

Document A discloses a 3'-end cDNA pool suitable for differential display from a small number of cells, wherein mRNA is reversed transcribed using a first primer population with a 3' degenerate base and a second primer population with four degenerate bases followed by PCR. The samples are diluted 25 times and used for differential display, wherein thermoresistant DNA polymerase is used for further amplification. The cDNA species are separated and characterised (see Abstract, Material and Methods and Table 1).

If the binding of the RNA polymerase is possible, then there should be a RNA polymerase binding site existent. The arguments that there is no reference in document A to a RNA polymerase *promoter* site cannot be accepted, because also in claim 1 there is no reference to it.

The additional features of claims 2-14 are also disclosed in document A (see Material and Methods, pages 844, 845 and table 1). Thus, these claims are also not novel (Article 33(2) PCT).

2.2 The method of claim 15 discloses additionally to the features of the method of claim 1, the incubation of the product with a restriction enzyme that specifically recognises the cleavage site in a restriction site included in the primer. These features are also disclosed in document A (see page 844) and therefore, claim 15 is also not novel (Article 33(2) PCT). The same applies to dependent claims 16-32, as their additional features are also disclosed in document A (see pages 843, 844,845,851).

The arguments given, relating to the fact that the restriction sites disclosed in document A are not considered to be *rare* restriction sites, as it is the case in claim 15, cannot be fully accepted as the term "rare" is relative and which enzymes are meant hereby cannot be clearly established.

2.3 Claim 33 relates to a heeled primer population. Claim 37 relates also to a heeled primer population, wherein the heel sequence includes a restriction site. Such a subject-matter is already disclosed in document A and therefore claims 33 and 37 are not novel (Article 33(2) PCT).

Document A discloses a degenerate primer population, which is not complementary to the mRNA molecules initially present in the sample, but has a region capable of hybridising with all the mRNA populations. The 3' primers consists of 14 (dT) and therefore anneals at the 3' end of poly(A) mRNAs. It also contains a degenerate base (V=A, C or G) as an anchor at its 3' end and the restriction site HindIII in the center, which overlaps with two dT bases. The 5' end of the primer is supplemented with nine-rich, arbitrarily selected bases, resulting in a 28-base total length. But also a 24- base length primer is used (see page 844 and table 2).

The additional features of claims 34, 35 and 39-40 are also disclosed in document A and therefore these claims are not novel (Article 33(2) PCT).

2.4 Claims 45-110 relate to methods similar to those claimed on claims 1 and 15, wherein the length of the nucleotides and amplification temperatures are given. Nevertheless, these features are also disclosed in document A (see pages 843-852) and therefore, these claims are also not novel (Article 33(2) PCT).

2.5 The subject-matter of claims 136-138 is also disclosed in document A (see pages 843 and 844) and therefore, these claims are also not novel (Article 33(2) PCT).

2.6 The subject-matter of claim 139, relating to a method to increase the number of nucleotide sequences corresponding to the mRNA species present in a sample, is not new in the sense of Article 33(2) PCT, because as already said

in 2.1, such a method is already disclosed in document A.

Document A discloses a 3'-end cDNA pool suitable for differential display from a small number of cells, wherein mRNA is reversed transcribed using a **first primer population with a 3' degenerate base and a second primer population** with four degenerate bases followed by PCR. The samples are diluted 25 times and used for differential display, wherein thermoresistant DNA polymerase is used for further amplification. The cDNA species are separated and characterised (see Abstract, Material and Methods and Table 1). If the binding of the RNA polymerase is possible, then there should be a RNA polymerase binding site existent.

The additional features of claims 140 and 141, relating to the existence of a rare cleavage site are also disclosed in document A (see page 844) and therefore, these claims are also not novel (Article 33(2) PCT).

3. Inventive step

3.1 Dependent claims 36 and 38 relate additionally to restriction enzymes, which are not disclosed in document A. However, it would be within the capabilities of the skilled person to select a different restriction site in the primer for a different restriction enzyme, without for that needing to perform an inventive step. In fact, the selection of an enzyme out of a group of known enzymes without a special reason for doing that and in the absence of unexpected effects, does not seems to involve an inventive step. Thus, these claims are not inventive (Article 33(3) PCT).

3.2 Claim 111 relates to a heeled primer population, wherein the difference between this subject-matter and the disclosure in document A, is the fact that the **heel sequence** have **15-22** nucleotides and the **oligo-dT sequence have 15-35 nucleotides**.

In document A the **heel sequence**, which is not complementary to the mRNA sequence, has **9 arbitrarily selected bases** and the **oligo-dT** sequence has **14** nucleotides.

This difference cannot confer inventiveness to claim 111, as it results in a merely new selection of ranges, without any particular effect. This possibility would be known to the skilled person, and therefore claim 111 is not inventive (Article 33(3) PCT).

The difference between the subject-matter of claim 118 and the disclosure in document a is the fact that the heeled primer population has a first variable sequence of 15-25 nucleotides (comparing with the 14 nucleotides disclosed in this document). For the same reasons as given for claim 111, this claim is also not inventive (Article 33(3) PCT).

Claims 112-117 and 119-128 do not contain any additional features which in combination with the features of the claim on which they depend, meet the requirements of the PCT in respect of inventive step (Article 33(3) PCT).

The additional features of claims 112-115, 117, 119-123 and 125-128 are already disclosed in document A (see pages 843-851) and therefore, known to the skilled person. Thus, these claims are not inventive (Article 33(3) PCT). The additional features of claims 116 and 124 are not disclosed in the prior art, but are known as possibility to the skilled person (see reasoning given in 3.1 of the present written opinion).

3.3 Claims 41-44 and 129-135, relating to a kit for the amplification of mRNA species present in a sample, are not inventive (Article 33(3) PCT), as it would be within the capabilities of the skilled person to put all the reagents needed for performing a known method together and obtain in this way the kit with the features disclosed in these claims.

4. Documents B and C are also relevant for the examination on inventive step of the claims of the present application:

- document B discloses the general amplification of target DNA by a single degenerate primer (see abstract)

- document C discloses a new primer strategy for improving precision of differential display, wherein ten-base nucleotides are added at the 5' end of the primers used in the initial differential display; these nucleotides include a restriction site to aid cloning (see abstract).

Re Item VI
Certain documents cited

1. The intermediate document cited in the International Search Report (WO 00 08208 A) will be of relevance for the examination on novelty and inventive step for the relevant parts of the present application if the date of priority is not validly claimed.

Re Item VII
Certain defects in the international application

1. A reference in claim 129 is wrong: " a first heeled primer according to any one of claims **11** to 116" should probably mean "...claims **111** to 116".

Re Item VIII
Certain observations on the international application

1. The applicant should be aware of the fact that the use of expressions like "preferably" (claim 1) and "optionally" (claim 45) has no limiting effect on the scope of the claim; that is to say, the feature following such expression is to be regarded as entirely optional (see Guidelines III-4.6).

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9 August 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/06004 A3

(54) Title: A METHOD FOR AMPLIFYING LOW ABUNDANCE NUCLEIC ACID SEQUENCES AND MEANS FOR PERFORMING SAID METHOD

(57) Abstract: The present invention relates to methods as well as to nucleic acid primers and kits containing the same for performing efficiently an amplification of nucleic acid sequences from a sample, particularly of nucleic acid sequences that are initially poorly represented in said sample.

INTERNATIONAL SEARCH REPORT

Inter. ..onal Application No
PCT/EP 00/06887

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, MEDLINE, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZHAO ET AL.: "3'-END cDNA POOL SUITABLE FOR DIFFERENTIAL DISPLAY FROM A SMALL NUMBER OF CELLS" BIOTECHNIQUES, vol. 24, May 1998 (1998-05), pages 842-852, XP002128314	1-9, 15-18, 24-30, 32, 45-54, 57,59, 61,63, 69-92, 95-97
Y	the whole document	10-14, 19-23, 31, 33-44, 55,56, 58,60, 62, 64-68, 93,94, 98-138
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Inter.. .onal Application No
PCT/EP 00/06887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	TELENIUS H ET AL: "DEGENERATE OLIGONUCLEOTIDE-PRIMED PCR: GENERAL AMPLIFICATION OF TARGET DNA BY A SINGLE DEGENERATE PRIMER" GENOMICS,US,ACADEMIC PRESS, SAN DIEGO, vol. 13, 1992, page 718-725 XP000199116 ISSN: 0888-7543 the whole document	1-138
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Y	DIXON ET AL.: "EXPRESSION PROFILING OF SINGLE CELLS USING 3 PRIME END AMPLIFICATION (TPEA) PCR" NUCLEIC ACIDS RESEARCH, vol. 26, no. 19, October 1998 (1998-10), pages 4426-4431, XP002128317 the whole document	1-138
Y	RICHARDSON ET AL.: "RELATING GENE EXPRESSION TO CELL FUNCTION USING A NOVEL SINGLE CELL PCR METHOD" EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 10, no. supp. '10!, August 1998 (1998-08), page 10307 XP000982404 the whole document	1-138
Y	FROUSSARD: "A RANDOM-PCR METHOD (rPCR) TO CONSTRUCT WHOLE cDNA LIBRARY FROM LOW AMOUNTS OF RNA" NUCLEIC ACIDS RESEARCH, vol. 20, no. 11, 1992, page 2900 XP002128315 the whole document	1-138
Y	WO 98 08973 A (HAMPSON LYNNE ;CANCER RES CAMPAIGN TECH (GB); HAMPSON IAN NOEL (GB) 5 March 1998 (1998-03-05) the whole document	1-138

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INTERNATIONAL SEARCH REPORT

Interr.. ..nal Application No
PCT/EP 00/06887

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEUNG AND NELSON: "WHOLE GENOME AMPLIFICATION USING A DEGENERATE OLIGONUCLEOTIDE PRIMER ALLOWS HUNDREDS OF GENOTYPES TO BE PERFORMED ON LESS THAN ONE NANOGRAM OF GENOMIC DNA" PNAS, vol. 93, 1996, pages 14676-14679, XP002128316 the whole document	1-138
Y	EBERWINE J ET AL: "ANALYSIS OF GENE EXPRESSION IN SINGLE LIVE NEURONS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 89, 1 April 1992 (1992-04-01), pages 3010-3014, XP002912945 ISSN: 0027-8424 the whole document	11,14, 40,44, 60, 127-129, 131, 133-135
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inten. .onal Application No
PCT/EP 00/06887

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